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**(54) Title:** THE PROTECTION OF EQUINES AGAINST STREPTOCOCCUS EQUI

**(57) Abstract**

A new bacterial vaccine to protect susceptible equine against *S. equi* which causes strangles. The vaccine stimulates a nasopharyngeal immune response in a susceptible equine through the presence of antibody activity in the nasopharyngeal mucus. The vaccine is a *S. equi* strain which contains an M protein fragment of 41,000 mw and is adapted for administration to equine either intranasally or orally as a vaccine. There is described a new strain of *S. equi* (709-27), a method of making and isolating useful vaccine strain of *S. equi* bacteria which stimulates an antibody response in the nasopharyngeal mucosa of the susceptible equine.

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TITLE

## The Protection Of Equines Against Streptococcus Equi

BACKGROUND OF THE INVENTION

15 This invention relates to the immunization of equines against Streptococci equi. S. equi causes strangles, an acute upper respiratory tract disease of horses, characterized by fever, nasal discharge and abscess formation in the retropharyngeal and mandibular lymph nodes. Horses that have been so infected in the field or experimentally infected with strangles and which do recover from strangles become highly resistant to reinfection. Moreover, only one antigenic type of S. equi has been observed in the field.

25 The above notwithstanding, vaccines prepared from bacterins of S. equi, or fractional extracts of the same, such as M protein-rich extracts, have been relatively ineffective to provide protection against S. equi in the field. This is true even though as far back as 1943 an article entitled "Studies with Equine Streptococcus" published in the Australian Veterinary Journal at 19:62 by P. O. Bazeley, presented a broad-range study of the problem coupled with test results which Dr. Bazeley and other characterized as very hopeful. However, many years 30 35 have passed without an adequate or effective method or

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1 means for protection of equines against strangles. One of  
the problems with earlier experimentation in the field was  
that scientists and researchers equated protection of the  
horse against S. equi with stimulation of bactericidal  
5 antibodies in the blood serum of the horse. In fact,  
vaccine failure was not due to failure of vaccines to  
stimulate bactericidal antibody in the serum, which it was  
shown did not equate with protection against field or  
experimental exposure to S. equi. In fact, it was dis-  
10 covered that ponies recently recovered from experimentally  
induced strangles were highly resistant to reinfection  
before serum bactericidal activity could be detected.  
Moreover, it was determined that the nasopharyngeal mucus  
of resistant ponies contained major IgG and IgA antibody  
15 activity against only one acid extract protein of about  
41,000 molecular weight (mw), whereas serum antibodies had  
a number of major specificities. These findings suggested  
that successful vaccination requires stimulation of the  
nasopharyngeal immune response.

20 The following publications have been made by the  
inventor herein relating to this development:

- 1) Abstract No. 172 appearing Abstracts IXth, Lancefield International Symposium on Streptococci and Streptococcal Diseases, Fuji, Japan, September 10, 1984;
- 25 2) Infection and Immunity, March 1985, Vol. 47, No. 3, pages 623-628;
- 3) Infection and Immunity, April 1985, Vol. 48, No. 1, pages 29-34.

30

#### BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a graph with separate coordinates for  
the IgA and IgG antibody titers in nasal washes of 14  
ponies against days which have passed following immuni-  
35 zation with S. equi 709-27. The antigens in the

3-

1 radioimmunoassay were acid extract and culture supernatant  
(the native form) protein of S. equi.

5 Figure 2 is a graph of cumulative mortality  
against days after challenge for groups of 40 mice vacci-  
nated with live S. equi 709-27, or an acid extract of S.  
equi 709-27 and a group of control non-immunized mice.  
All mice were challenged with  $5 \times 10^{-7}$ -CFU virulent S.  
equi CF32. The information of Fig. 2 is important because  
it shows that S. equi 709-27 carries the intact M protein,  
10 similar to that of the parent S. equi CF32.

15 Figure 3 is an immunoblot showing proteins (SDS  
PAGE), S. equi and S. zooepidemicus recognized by IgG and  
IgA in nasopharyngeal mucus and in serum of a pony follow-  
ing intranasal vaccination with S. equi 709-27. The blots  
were washed in monospecific antisera against equine IgA or  
IgG following treatment with nasopharyngeal mucus or serum.

Tracks: A - Acid extract of S. zooepidemicus  
B - Acid extract of S. Equi (CF32)  
C - Culture supernatant protein of S. equi (CF32)

20 Antigens of S. zooepidemicus were included because  
most horses carry this organism in the nasopharynx and  
therefore are stimulated to make antibodies to its  
proteins, some of which are common to S. equi.

25 The measurement technique described in the  
Figures are similar to those discussed in the following  
publications:

- a) Infection and Immunity Vol. 47, No. 3 pages  
623-628 (March 1985);
- b) Infection and Immunity Vol. 48, No. 1 pages  
30 29-34 (April 1985).

#### DESCRIPTION OF THE INVENTION

35 The present invention teaches how to stimulate  
the nasopharyngeal immune response, for example using a  
bacterial clone derived from a highly virulent strain of  
S. equi known as S. equi CF32 which is on deposit at the

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1 American Type Culture Collection, (A.T.C.C. No. 53185)  
Rockville, Maryland, and available to the public as of the  
time this patent application is issued. CF32 produces  
large (1-3 mm) transparent, mucoid colonies that tend to  
5 flow together and are surrounded by a wide zone (5-10 mm)  
of beta hemolysis. Also on deposit with the American Type  
Culture Collection is a derivative of S. equi CF32 which  
has been rendered avirulent according to the teachings of  
the present invention. The avirulent derivative S. equi  
10 bacterium is known as Cornell S. equi 709-27 and will be  
available through the A.T.C.C. under A.T.C.C. No. 53186  
when this patent application issues as a U.S. Patent.  
Cornell 709-27 produces a small (0.5 mm in diameter white,  
15 convex smooth surfaced colony surrounded by a narrow (1  
mm) zone of beta hemolysis.

This invention relates to an equine vaccine  
against S. equi caused strangles in an equine, which  
vaccine stimulates a nasopharyngeal immuno response in a  
strangles susceptible equine and which vaccine comprises  
20 an avirulent strain of S. equi formed by mutating a  
virulent strangles causing S. equi strain to render it  
avirulent while retaining thereon protein which provides  
an acid extract M protein fragment with a molecular weight  
of about 41,000 which stimulates an immunological response  
25 to IgG and IgA antibody similar to that in the nasopharyn-  
geal mucus of an equine recovered from S. equi caused  
strangles.

The vaccine of the invention is not strain  
specific. Only one antigenic type of S. equi has been  
30 observed in the field. Thus, the method of the invention  
can be applied to any virulent strangles causing S. equi  
strain.

The virulent S. equi strain can be rendered  
avirulent in any manner so long as the resultant avirulent  
35 S. equi strain retains the M protein fragment having a

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1 molecular weight of about 41,000 which stimulates an  
immunological response similar to that in the nasopharyn-  
geal mucus of an equine recovered from S. equi caused  
strangles. The presently preferred method is deliberately  
5 induced mutagenesis for example by the use of chemicals or  
radiation. Particularly useful is chemical mutagenesis  
for example through the use of nitrosoguanidine. (See  
Chapter 13, Gene Mutation, Manual of Methods of General  
Bacteriology, American Society for Microbiology,  
10 Washington, D.C. 1981).

For the purposes of characterizing the vaccine of  
the invention through radio-immunoassay or immunoblotting  
assay the acid extract protein is isolated following  
techniques described in a publication by R.C. Lancefield  
15 entitled "The Antigenic Complex of Streptococcus  
Hemolyticus I Demonstration of a Type Specific Substance  
in Extracts of Streptococcus Hemolyticus" J. Exp. Med.  
47:91.

20 For the purpose of further characterizing the  
vaccine of the invention protein molecular weight is  
determined by SDS - PAGE Electrophoresis and the use of  
molecular weight standards.

25 In accordance with the teachings of the present  
invention a successful vaccine against S. equi requires  
stimulation of the nasopharyngeal immune response in a  
susceptible equine by intranasal or oral inoculation.  
Antibody activity in the nasopharyngeal mucus correlates  
with protection against strangles, and antibody activity  
in the blood serum is of less significance.

30 M-protein-rich extracts were relatively ineffec-  
tive because they did not stimulate a nasopharyngeal  
immune response of the susceptible equine, although they  
were effective in producing an immune response in the  
blood serum of the animal. In order to stimulate the  
35 required response the present invention teaches a method  
of making avirulent S. equi bacteria which may be used as

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1 a vaccine and applied either intranasally or orally and  
produces major IgG and IgA antibody responses in the  
nasopharyngeal mucus of the susceptible equine. The  
avirulent strain of S. equi (Cornell 709-27) is such a  
5 bacteria. Hereinafter that especially made bacteria is  
called S. equi (Cornell 709-27).

Method of Producing an Effective Avirulent  
Vaccine Strain of S. equi

10 The strain of S. equi (Cornell 709-27) avirulent  
for mice and ponies was obtained in the following manner:  
the starting bacteria, S. equi CF32 was subjected to  
nitrosoguanidine mutagenesis following the teachings set  
out in an article by Carlton, B.C. and Brown, B.J. (1981)  
15 in Manual of Methods for General Bacteriology. (Eds. P.  
Gerhardt, et al.) American Society for Microbiology,  
Washington, D.C., p. 226. Modification of the procedure  
set forth in the first column of page 226 was undertaken.  
Specifically, Todd Hewitt broth was used throughout the  
20 procedures as a growth medium. Nonencapsulated colonies  
were screened for loss of virulence by intraperitoneal  
inoculation of mice (ICR). The strains which did not kill  
mice were considered positive strains. The positive mouse  
strains were then used to vaccinate mice by the intraperi-  
25 toneal route to determine their protective quality. Those  
strains which were protective of mice were inoculated  
intranasally into horses. Finally, as described herein, a  
derived strain of S. equi 709-27 was found to be avirulent  
in a dose of  $3 \times 10^9$  CFU, and efficacious as a vaccine  
30 against S. equi in susceptible equine when it was intra-  
nasally or orally inoculated in the equine. Moreover, the  
positive strain which also protected equines tested for  
the presence of the 41k fragment of the M protein by  
immunoblotting. The identifying number for that strain is  
35 S. equi 709-27.

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1 An acid extract of strain S. equi (Cornell 709-27)  
 was shown by immunoblotting to carry the same immunologically reactive proteins as the parent S. equi strain  
 5 (CF32). The immunoblotting procedure used was similar to that used in a scientific article entitled "Infection and Immunity" Vol. 48, No. 1 pages 29-34 (April 1985).

Equine Immunization and Challenge

10 The S. equi (Cornell 709-27) was then tested for efficacy as a vaccine against experimental S. equi infection in equine. The following table depicts that testing.

15 Table 1. Resistance of Ponies to Intranasal Challenge with Streptococcus equi Following Intranasal Immunization with the Avirulent Strain of S. equi 709-27.

	Treatment (vaccinate with <u>S. equi</u> 709-27)	Challenge (CFU Virulent <u>S. equi</u> CF32)	No. Ponies	No. Resistant
20	$3 \times 10^9$	$5 \times 10^8$	14	14
25	Day 0 and Day 30 Contact Controls Isolation Controls	Day 59 " "	2 2 6	2 2 0*

30 \*All controls developed acute strangles within 4 days of challenge.

Fourteen yearling ponies raised in isolation and never exposed to S. equi were given an atomized suspension (intranasally) of an 18 hour culture ( $3 \times 10^9$  CFU) in Todd Hewitt broth of 709-27. A repeat inoculation was given 29 days later. Ponies were challenged intranasally 30 days

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1 later with  $5 \times 10^8$  CFU of an overnight culture of S.  
equi CF32. Cultures were administered by means of a nasal  
atomizer (Model #281, Devilbiss Co., Somerset, PA).

5 Six non-vaccinated ponies housed separately from  
the vaccinated group and 2 contact control ponies were  
also challenged with the same CF32 inoculum. All of the  
immunized ponies and the 2 contact control ponies were  
resistant to S. equi when challenged, but all of the  
isolation controls developed acute strangles within 4 days  
10 of challenge.

In addition about 800 horses on farms with  
endemic S. equi infection problems were experimentally  
intranasally or orally vaccinated with S. equi 709-27 to  
date and only two horses have developed strangles. The  
15 expected occurrence of strangles on those farms based on  
the experience of the three previous years, is such that  
one would have predicted the occurrence of strangles in  
the range of 40% of the horses.

When using the teachings of the present invention  
20 to vaccinate horses against S. equi the results of oral  
inoculation appeared to be comparable with intranasal  
inoculation with the same dose. The vaccine dose (number  
of organisms) used in the vaccination described herein was  
100 times greater than the number of organisms of a wild  
25 virulent strain of S. equi (CF32), which would be expected  
to cause disease in a normal equine. However, a commer-  
cial S. equi vaccine program would utilize dosage levels  
which were determined by consultation between the manufac-  
turer and the appropriate governmental authorities.

30 Freezing or freeze drying does not adversely  
affect the vaccine. These procedures can therefore be  
used in mass production and distribution of the vaccine.

The vaccine has been entirely without side  
35 effects in adult animals, but a low (-5%) incidence of  
submandibular abscesses has been observed on one occasion  
in 3-month foals. This adverse reaction occurred when a

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1 very heavy dose of vaccine was administered in an effort  
to obtain consistent seroconversion in the blood serum of  
the inoculated equine. As stated elsewhere, it is  
5 nasopharyngeal mucus of the susceptible equine that  
contains antibodies involved in immunological protection.

Antibody Assays - Figure 1

IgA and IgG antibodies to the proteins of S. equi  
(CF32) were assayed in sera and nasal washes collected  
10 before, during, and after vaccination and challenge.  
Assays were performed by solid phase radioimmunoassay as  
described in an article entitled "Immunochemical Quan-  
titation of Antigens by Single Radial Immunodiffusion"  
by G. Mancini, A.O. Carbonara and J.H. Heremans in  
15 Immunochemistry 2: pages 235-254. Wells were coated with  
acid extract (AE) or culture supernatant (CS) protein of  
S. equi.

IgA and IgG antibody responses to acid extract  
and culture supernatant proteins of S. equi were observed  
20 in nasal washes from all vaccinated animals (Figure 1).  
Serum antibody responses were also observed, but they were  
inconsistent. Contact control ponies showed nasal and  
serum antibody conversion at the same time - an indication  
25 that transmission of the vaccine strain had occurred in  
the group. Principal and contact control ponies were  
resistant to challenge with virulent S. equi whereas  
non-vaccinated ponies developed typical strangles within 4  
days of challenge (Table 1).

30

Mouse Immunization and Challenge - Figure 2

The mouse has historically been the model for the  
immunology of S. equi infection. Accordingly, as a  
parallel test of efficacy, adult ICR mice were immunized  
35 subcutaneously with hydroxyapatite purified protein of an  
acid extract of S. equi 709-27. Reference is made to an  
article by Vosti, K.L. Journal of Med. Microbiol. 11:453

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1 (1978). Protein was absorbed to aluminum hydroxide and  
administered in two subcutaneous doses of 50 µg 21 days  
apart. All mice, including a group of non-vaccinated  
controls, were later challenged with virulent S. equi (5 x  
5  $10^7$  CFU) given intraperitoneally. Mouse mortality was  
recorded for 7 days following challenge. The difference  
in mortality between the control and vaccinated groups was  
highly significant using the Chi square analysis used in  
statistics. The mice immunized either with an acid  
10 extract or live cells of S. equi 709-27 showed a signifi-  
cant protective response (probability  $\leq .01$ ) as compared  
with non-vaccinated controls (Figure 2). This result  
suggested that S. equi 709-27 retained the protective M  
antigen of S. equi.

15 Notwithstanding the fact that it is not virulent,  
an acid extract of S. equi 709-27 was shown by immuno-  
blotting to carry the same immunologically reactive  
proteins as the parent S. equi strain.

20 Fig. 3 Immunoblotting Showing Proteins Recognized  
by Mucosal and Serum Antibodies

25 The immunologically reactive proteins in an acid  
extract and culture supernatant of S. equi and an acid  
extract of S. zooepidemicus were distinguished on nitro-  
cellulose blots of SDS - PAGE gels. Blots were treated  
with sera or nasopharyngeal mucus collected when ponies  
were killed 7 days after challenge. A scientific article  
entitled "Infection and Immunity" Vol. 47, No. 3 pages  
623-628 (March 1985) describes the technique used.

30 Immunoblotting revealed that IgA and IgG  
antibodies in nasopharyngeal mucus of vaccinated animals  
were directed mainly against a 41k M protein fragment,  
whereas serum antibodies had a much broader spectrum of  
activity, a finding noted previously in ponies following  
35 recovery from experimentally induced strangles. Since an  
antibody response to the 41,000 mw M protein fragment is a

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1 constant feature of the nasopharyngeal immune response of  
resistant horses, it is an important protective antigen.

2 The antibody response is also specific to S. equi  
because similarly reactive proteins of S. zooepidemicus  
5 could not be detected on the immunoblot (Track A). Other  
studies have indicated that antibodies in strongly  
bactericidal sera react strongly with M protein fragments  
of about 29,000 and 37,000 molecular weight. A hypothesis  
10 to explain the different molecular weights of the M  
protein fragments of S. equi recognized by serum and  
nasopharyngeal antibody is that the portion or region of  
the M protein molecule of S. equi important in the  
nasopharyngeal response, differs from that involved in the  
stimulation of bactericidal antibody in serum.

15 In summary, the present invention teaches a new  
and improved bacterial vaccine to protect susceptible  
equine against S. equi which causes strangles. The  
vaccine stimulates a nasopharyngeal immune response in a  
susceptible equine through the presence of antibody in the  
20 nasopharyngeal mucus. The vaccine is a S. equi strain  
which contains an M-protein fragment of 41,000 mw and is  
adapted for administration to equine either intranasally  
or orally as a vaccine. The teachings of the present  
invention include: a new strain of S. equi (709-27), a  
25 method of making and isolating useful vaccine strain of S.  
equi bacteria, and which stimulates an antibody response  
in the nasopharyngeal mucosa of the susceptible equine.

30 Accordingly, it is to be understood that the  
embodiments of the invention herein described are merely  
illustrative of the application of the principles of the  
invention. Reference herein to details of the illustrated  
embodiments are not intended to limit the scope of the  
claims which themselves recite those features regarded as  
essential to the invention.

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1 I CLAIM:

1       1. A vaccine for protecting equines against S. equi caused strangles which comprises an avirulent strain of S. equi which stimulates an antibody response in the 5 nasopharyngeal mucosa of the susceptible equine.

10       2. A vaccine as in Claim 1 against S. equi caused strangles in an equine, which vaccine stimulates a nasopharyngeal S. equi antibody response in a strangles susceptible equine and which vaccine comprises an avirulent strain of S. equi formed by mutating virulent strangles causing S. equi strain to render it avirulent while retaining thereon protein which provides an M 15 protein fragment with a molecular weight of about 41,000 which stimulates an immunological response in the form of IgG and IgA antibodies in the nasopharyngeal mucus of an equine similar to that found in an equine which has recovered from S. equi caused strangles.

20       3. The vaccine of Claim 1 wherein the strain avirulent S. equi is nonencapsulated and includes an M-protein fragment with a molecular weight of about 41,000.

25       4. The vaccine of Claim 3 wherein the strain of avirulent S. equi is S. equi 709-27.

30       5. The vaccine of Claim 1 wherein the strain of avirulent S. equi includes an M-protein fragment with a molecular weight of about 41,000 and which can be inoculated intranasally or orally.

35       6. The vaccine of Claim 5 wherein the strain avirulent S. equi is S. equi 709-27.

30       7. A method of protecting equines against avirulent S. equi which comprises inoculating an equine either intranasally or orally with a strain of avirulent S. equi which stimulates a nasopharyngeal antibody response in the nasopharyngeal mucosa of a susceptible equine.

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- 1                   8. The method of Claim 7 wherein the strain of S. equi is both avirulent and carries at least M-protein fragments of about 41,000 mw.
- 5                   9. The method of Claim 8 wherein the strain avirulent S. equi is S. equi 709-27.
- 10                  10. A vaccine for protecting equines against S. equi which comprises an avirulent strain of S. equi known as S. equi 709-27 which can be inoculated intranasally or orally in the susceptible equine.
- 15                  11. A method of making a strain of S. equi which is avirulent for equines comprising of the following steps:
  1. subjecting a virulent strain of S. equi to mutagenesis;
  2. selecting a resulting bacterium which provides an M protein fragment having a molecular weight of 41,000, which bacterium produces an S. equi antibody response in the nasopharyngeal mucus of an S. equi susceptible equine.

20

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FIG. 1

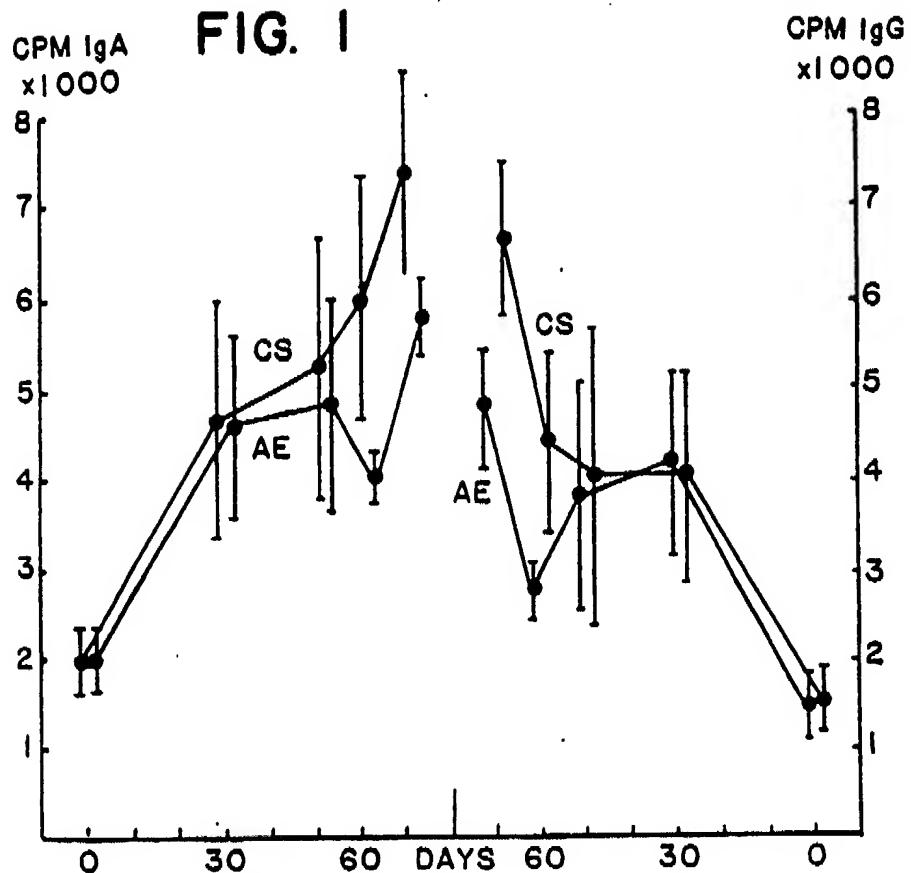


FIG. 3

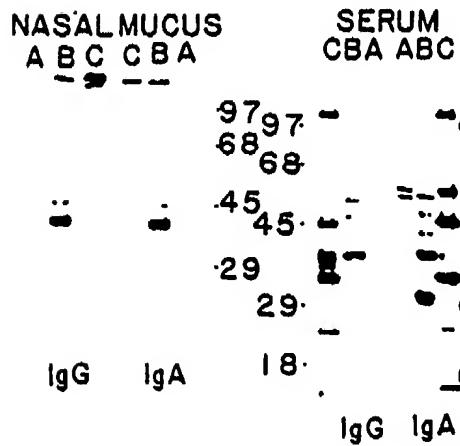
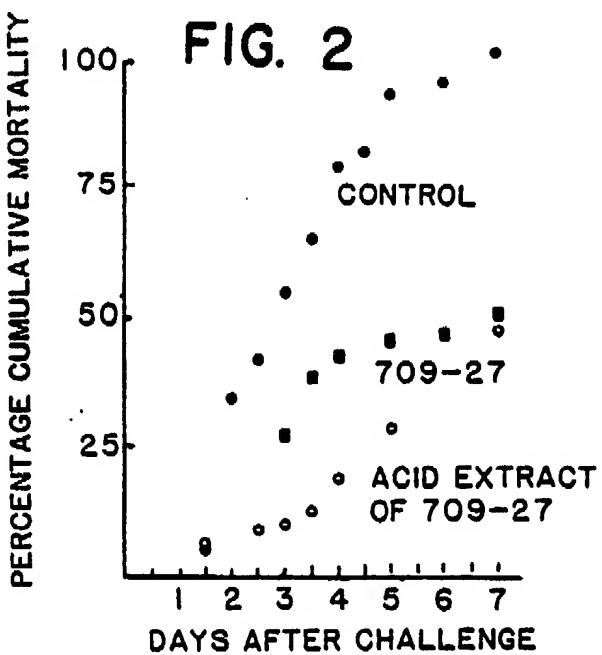


FIG. 2



# INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/01460

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) <sup>1</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC: A61K 39/09; C12N 15/00; 1/20  
 U.S.: 424/92,93; 435/172.1, 253

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>4</sup>

Classification System	Classification Symbols
U.S.	424/92, 93; 435/172.1, 253

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched <sup>5</sup>

ONLINE COMPUTER SEARCH CHEMICAL ABSTRACTS 1967-1986;  
 BIOSIS 1967-1986. SEARCH TERMS: STREPTOCOCCUS EQUI,  
 ATTENUATED STRAINS AND VACCINES.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>

Category <sup>6</sup>	Citation of Document, <sup>15</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>16</sup>
A, P	US, A, 4,582,798 (BROWN) 15 April 1986. See entire document.	1-10
A	Infection and Immunity, Volume 48, No. 1, issued 1985 (U.S.A.), J. F. Timoney, "Immunologically Reactive Proteins of Streptococcus equi". See pages 29-34.	1-10
A	Infection and Immunity, Volume 47, No. 3, issued 1985. (U.S.A.), Jorge E. Galan, "Mucosal Nasopharyngeal Immune Response of Horses to Protein Antigens of Streptococcus equi". See pages 623-628.	1-10

\* Special categories of cited documents: <sup>15</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search <sup>8</sup>

10 September 1986

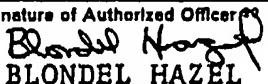
Date of Mailing of this International Search Report <sup>9</sup>

23 SEP 1986

International Searching Authority <sup>10</sup>

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Signature of Authorized Officer <sup>11</sup>

  
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